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Full Length Research Paper

Serum immunoglobulins (IgA, IgM, IgE) and acetylcholinesterase level determination in agricultural spray workers exposed to organophosphates pesticides in district Gujranwala

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Background: Acetylcholinesterase is enzyme instantly performs the hydrolytic cleavage of acetylcholine which is a neurotransmitter. Enzymatic degradation of neurotransmitter was inhibited by organophosphates exposure and in activation or decrease levels of acetylcholinesterase leads to accumulation of excessive acetylcholine which resulted in paralysis, respiratory failure and even death. Methods:Organophosphate impact on serum immunoglobulins (IgA, IgM and IgE) and acetylcholinesterase level was determined in pesticides exposed local agricultural spray workers (n= 200) and health control group of pesticides unexposed persons (n= 100). Results: Acetylcholinesterase level mean value in male spray workers (0.16 U/ mL; -0.10-0.66 U/ mL) and non-spray male workers (0.44 U/ mL; 0.01-0.93 U/ mL) was highly significant at p>0.05. Similarly, highly significant difference at p>0.05 was observed in acetylcholinesterase level mean value of female spray workers (0.41 U/ mL; 0.06-0.93 U/ mL) and non-spray female workers (0.65 U/ mL; 0.32-1.1 U/ mL). Conclusion: In current research it was observed that serum acetylcholinesterase level was reduced in the male and female spray workers as compared to non-spray workers. Mean high levels of IgA, IgM and IgE immunoglobulins were observed in both male and female spray workers. Hence it is concluded that organophosphates exert toxic impact on human health. So use of these pesticides must be minimized.

Keywords: Acetylcholinesterase, Immunoglobulins, Organophosphate pesticides, ELISA

INTRODUCTION

Occupational and environmental exposure to pesticides caused range of serious human health problems. Annually 10,000 deaths due to use of pesticides was estimated worldwide with about three fourth was occurred in developing countries (Horrigan *et al*., 2002). Exposure to pesticides results in potential risk of acute and chronic health problems for spray workers as compared to general population exposed to traces of pesticides indirectly by

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pesticides contaminated food and water (Yassi *et al*., 2001; Amer, 2002).

In agriculture sector of developing countries pesticides are extensively used. However, to save crops from the pests attack and to increase the crop production diverse group of various costly and hazardous, synthetic pesticides are used. Among the population of Pakistan 70% of the population lives in the villages and are mostly dependent on agricultural productivity directly or indirectly (Kamel and Hoppin, 2004; Mnif *et al*., 2011). The extensive use of pesticides i.e., organophosphates have great concern due to health hazards in human beings as well as wild and domestic animals (Alpalan *et al*., 2006). Exposure to pesticides is associated with serious health problems including metabolism impairment, neurotoxicity, carcinogenicity, reproductive and endocrine disruption as well as immune dysfunctions (Bolognesi, 2003). Due to exposure these pesticides results mortality and morbidity in most of the less developed countries of the World such as Pakistan, India and Bangladesh (Saxena, 2010).

The Acetylcholinesterase (AChE) is enzyme instantly performs the hydrolytic cleavage of acetylcholine, responsible for the physiological transmission of nerve action potential. Nearly all organophosphates insecticides cause toxic effects in humans through the inhibition of AChE in the nervous system (Costa *et al*., 2005). The organophosphates compounds such as malathion, diazinon and dichlorvos toxicity was determined in plasma by cholinesterase level as reported by different workers (Hernandez et al., 2006; Jintana *et al*., 2009). Duration of exposure as well as type of pesticides used was also implicated in a significant variation of cholinesterase activity and can be considered as risk factors of exposure to pesticides. AChE levels assessment is better to analyze the cumulative inhibition caused by chronic exposure to organophosphates insecticides (Araoud *et al*., 2011).

ACh is the chemical transmitter of somatic motor neurons to skeletal muscle, postganglionic parasympathetic nerve fibers, preganglionic fibers of both sympathetic and parasympathetic nerves, and some fibers in the central nervous system. The acute toxicity, initiated by the inhibition of the acetylcholinesterase enzyme (AChE) with the subsequent accumulation of acetylcholine (ACh) in the nervous termination, provoking an overstimulation of muscarinic acetylcholine (mAChR) and nicotinic acetylcholine (nAChR) receptors (Vittozzi *et al*., 2001). The accumulation of ACh at the motor nerves results in weakness, fatigue, muscle cramps, fasciculations, and muscular weakness of respiratory muscles. Accumulation at the autonomic ganglia results in increased heartbeat and blood pressure, pallor, and hypoglycemia. Accumulation of ACh at muscarinic receptors results in visual disturbances, tightness in the chest and wheezing due to bronchoconstriction and increased bronchial secretions, and increased salivation, lacrimation, sweating, peristalsis (resulting in nausea, vomiting, cramps, diarrhea), and urination. (Costa, 2006).

Immune system is the first defense line against pathogenic organisms; however, it is altered by the vulnerable environmental factors such as OPs, which can cause structural or functional alterations in humoral or cell mechanisms (nonspecific or adaptative) of the immune response which entails, among others, an increase in the susceptibility to infections (Li *et al*., 2013).

Correlation between various enzymes especially with acetylcholinestrase and harmful effects of pesticides was reported by many researchers (Ahmed and Mohammad, 2005; Remor *et al*., 2009; Vrioni *et al*., 2011; Dias *et al*., 2013). Measurements of AChE as primary biomarker in case of clinical and accidental organophosphates poisoning was reported by many researchers (Ng *et al*., 2009; Ueyama *et al*., 2010). However, very little work has been done on this aspect in Pakistan. Therefore, in the current research analysis of blood samples of agriculture spray workers compared with non-agricultural workers as control was carried out to find the impact of organophosphates pesticide exposure on serum acetylcholinesterase and immunoglobulins (IgA, IgM and IgE) levels.

MATERIALS AND METHODS

The agriculture spray workers included in the current research work were working in the fields from quite long duration i.e., above ten years. Before the blood sampling, written consent from all the subjects were taken. All the phlebotomy procedures were carried out as described by WMA (2008). Fresh blood sample (5 ml) was drawn into vacutainer tubes from each of the spray workers (n= 200) and the unexposed healthy control individuals (n= 100). About 2.5 ml blood was dispensed into serum separation tube containing gel in order to separate serum from the blood for biochemical tests. The isolated serum samples were stored at -70^{0}C for subsequent biochemical tests.

Biochemical analysis

Determination of acetylcholinesterase activity

Assay of acetylcholine in a total volume of 200 µL per micro plate well was carried out. Acetylcholine standard curve was prepared by using 100 mM acetylcholine stock solution prepared into 1X reaction buffer. 1X Reaction buffer (1X) without acetylcholine was used as a negative control. Working solution of 400 µM Amplex Red reagent was prepared and reactions were started after adding 100 µL of the Amplex Red reagent/HRP/choline oxidase/acetylcholinesterase working solution to each microplate well containing the samples and controls.

Table 1: Acetylcholinesterase levels determination in male spray workers (n= 150).

Category	N	Mean Unit value (U/mL)	Std. Deviation	Median	Std. Error of Mean	Min	Max	Range	Mean Square	F	Sig.
Spray workers	150	0.16	0.22	0.08	0.02	-0.10	0.66	0.76	2.81	62.3	0.00**
Non spray workers	50	0.44	0.19	0.43	0.03	0.01	0.93	0.93	0.045		

**highly significant (p>0.05)

Table 2. Acetylcholinesterase levels determination in female spray workers (n= 50).

Category	N	MeanUnit value (U/mL)	Std. Deviation	Median	Std. Error of Mean	Min	Max	Range	Mean Square	F	Sig.
Spray workers	50	0.41	0.24	0.08	0.03	0.06	0.93	0.87	1.42	16.31	0.00**
Non spray workers	50	0.65	0.33	0.43	0.04	0.32	1.63	1.31	0.087		

**highly significant (p<0.05)

Reactions were incubated for 30 minutes and protected from light. Fluorescence was measured in a fluorescence micro plate reader using detection at 560 nm.

Determination of levels of immunoglobulins

The serum concentration of immunoglobulins was measured by sandwich ELISA. The microtitter plates were coated with antibodies at concentration of 5µg/mL in coating buffers (.05M bicarbonate buffers, pH 9.6). Washing of coating solution was carried out by using 200 µL 1xPBS (phosphate buffer saline) and .05% tween 20. Remaining protein binding sites were blocked by adding 200 µL of 5% BSA/PBS per well. Plates were incubated again for 1 to 2 hours at room temperature. Then 100 µL of serum samples were added to each well and incubated again for one hour. Then incubated again for one hour after adding 100 µL secondary antibody and HRP (horse raddish peroxidase). Washing was carried out four times with PBS. 100µL blocking buffer was added to stop the reaction and washing was performed. Then absorbance was measured at 450nm on ELISA reader.

RESULTS

Biochemical analysis of acetylcholinestrase

The activity level of serum acetylcholinestrase, standard deviation, and minimum and maximum range in control group and various spray workers exposed to different pesticides was analyzed. As the cumulative effect of various pesticides in spray workers depression in acetylcholinestrase activity was found to be quite significant in each category of spray workers.

Analysis of level of acetylcholinestrase concentration in blood serum of male spray workers

Serum acetylcholinesterase activity level of male spray workers analyzed by ANOVA (SPSS version 21) results the highly significant difference (p>0.05) The mean acetylcholinestrase level in the male spray workerss was (0.16 U/mL) and in non-spray workerss was (0.44 U/mL) as shown in **Table 1**.

Analysis of level of acetylcholinestrase concentration in blood serum of female spray workers.

It was found in this research that pesticides toxicity become cause of depressed activity activity level of acetylcholinestrase in spray workers who were exposed to various groups of organophosphates while spraying in fields. Results analyzed by ANOVA (SPSS version 21) indicated that the highly significant reduced level of acetylcholinestrase was (*p*>0.05) found among spray workers and non spray workers. The mean cholinestrase level in the female spray workers was (0.41 U/mL) and in female non spray workers was 0(.65 U/mL) as shown in **Table 2.**

Acetylcholinestrase standardization

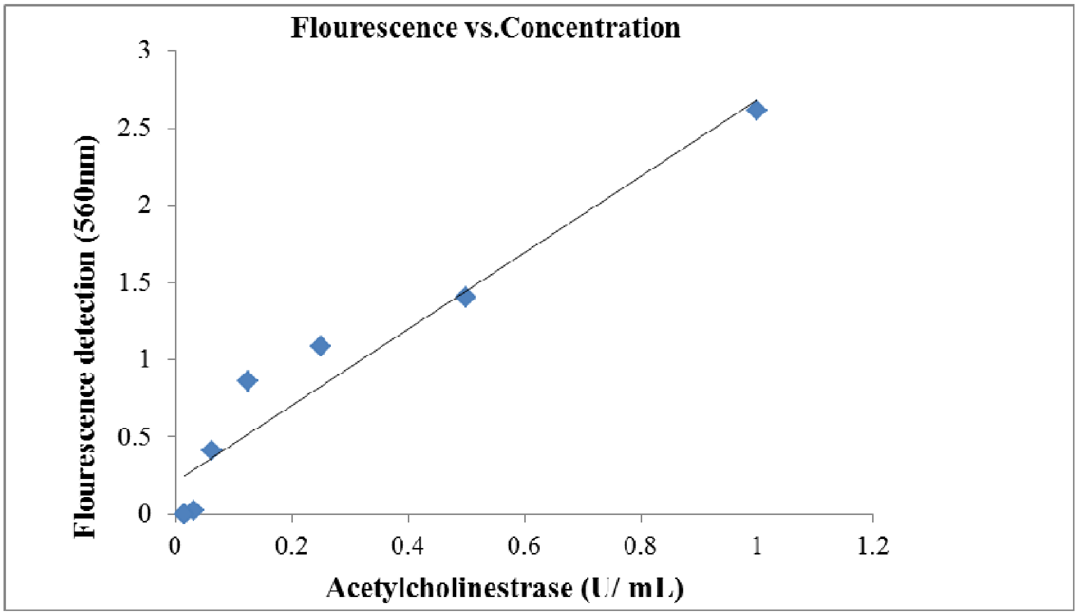

Figure 1: Standard curve between acetylcholinestrase different concentrations (1-0.015 U/mL) and fluorescence detected by fluorescence microplate reader at 560nm.

a) Graphical comparison of mean acetylcholinestrase values between male spray workers and non-sprayer worker. (b) Graphical comparison of mean acetylcholinestrase values between female sprayer and non-sprayer workers. (c) Graphical comparison of acetylcholinestrase values between male sprayer and
female sprayer workers

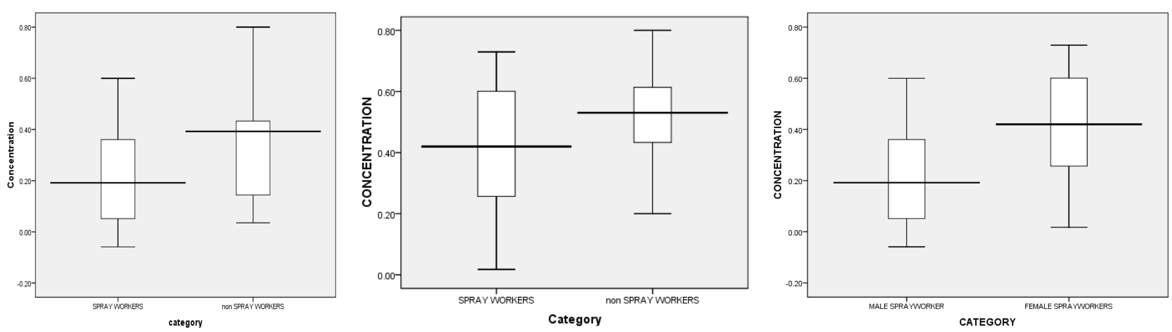

Figure 2: Box-Whisker Plots-Graphical comparisons of mean acetylcholinestrase values between male/female sprayer & non-sprayer workers and between male & female spray workers

Immunoglobulins concentration in blood serum of male spray workers

The level of various immunoglobulines is shown significance differences of immunoglobulines (IgA, IgM, IgE). Serum immunoglobulines levels mean values of male spray workers and non spray workers as compares to the reference values. The concentration of immunoglobulinesI gA and IgM were significantly different in male spray workers as compared no male non spray workers but but IgE was not significantly different**.** Serum immunoglobulines concentration mean values of spray workers and non spray workers were indicated that immunoglobulines IgA and IgM were significantly different in spray workers as compared no non spray workers but IgE was not significantly different in both male and female groups as shown in **Table 3.**

Table 3: Levels of immunoglobulins in blood serum of male spray workers

Spray workers							Non spray workers					
Parameter (Units)	**Gender**	**Normal range**	**Mean values** (mg/dL)	**Mean square**	**Min-Max**	**S.D**	**Mean values** (mg/dL)	**Mean Square**	**Min-Max**	**S.D**	***df***	**p-value**
IgA (mg/dL)	Male	70-400	350.3	5512.6	201-440	36.7	341	1154.8	302-332	9.1	1	0.033**
	Female		348.76	18117	299-440	26.29	336	724	201-325	28.9	1	0.000**
IgM (mg/dL)	Male	40-230	157	6093	108-171	16.1	119	225.5	103-123	6.0	1	0.000**
	Female		161.6	9400	108-182	17.2	124.6	248	103	139	1	0.000**
IgE (mg/dL)	Male	<.023	0.027	0.001	0.01-.027	0.035	0.019	0.000	0.02-0.14	0.03	1	0.928
	Female		0.032	0.001	0.01-0.28	0.04	0.015	0.645	0.01	0.14	1	0.425

**highly significant (p<.05) Note: The normal rage of reference values in healthy individuals as mentioned by (Aroonvilairat *et al*., 2015)

DISCUSSION

To determine the levels of organophosphate pesticides in blood serum of spray workers in comparison with non-spray workers is essential to determine toxicity of these pesticides. Manufacturers are compelled to create and mass produce effective yet less toxic pesticides. Inhibition of enzymatic degradation of neurotransmitters due to organophosphate pesticides exposure resulted into disturbed function of neurons which ultimately leads to paralysis, respiratory failure and death as reported by several workers (Aygun *et al*., 2002).

Acetylcholinesterase was used as an index for chronic exposure to organophosphate pesticides in spray workers in the current research. Quantitatively decreased level of acetylcholinesterase was assessed in pesticides exposed spray workers than non-spray workers. Significant difference in acetylcholinesterase levels observed in male and female spray workers to non-spray workers at *p*= 0.000 as shown in **Table 1 and 2**. In male spray workers acetylcholinesterase was (0.16 U / mL) and in non-spray workers (0.44 U/mL) while in female spray workers acetylcholinesterase was (0.41 U/mL) and in non-spray female workers (0.65 U/mL) which is highly significant at p= 0.000 as described in current research. This indicated that spray workers were at higher health risk associated with organophosphate pesticides exposure. Thus, decreased acetylcholinesterase level observed in spray workers which is one of the most important enzymes required for the proper function of nervous system. This finding is in agreement with (Woreka *et al*., 2004) who reported the same fact while working on serum samples of pesticides exposed workers. The enzyme acetylcholinesterase is responsible for the expedient breakdown of the neurotransmitter acetylcholine. Due to organophosphates exposure the normal transmission of a nervous impulse in spray workers nervous system fired resulting in uncoordinated muscle movement, nausea, dizziness, and eventually seizures and unconsciousness. In previous study conducted by More *et al*. (2003) showed that spray personnel without protective clothing presented a marginal reduction in their blood cholinesterase activity during the exposure period. Due to long term organophosphates exposure acetylcholinesterase enzyme inhibition resulted in impairment of sensory and motor nerve conduction was reported in spray workers of Asian countries including Srilanka and India (Smit *et al.,* 2003; Karabay *et al*., 2004; Hernandez *et al*., 2006; Kesovachandran *et al*., 2006).These finding were in agreement with current research findings. In Pakistan, study on organophosphates pesticides exposure with ultimate simultaneous effect on acetylcholinesterase enzyme levels and immunoglobulins levels in spray workers was not carried out earlier and hence current research findings provided information about these aspects in spray workers.

This study revealed that pesticides cause immunotoxicity in spray workers. It was analyzed that immunoglobuline IgA and IgM (*p*=0.033 and *p*=0.000) in male spray workers and non spray workers were significan respectively. Similarly significant difference of immunoglobulin IgM and IgA *(p* =0.000 and *p*=0.000) in female spray workers and non spray workers was also observed. Non significant difference was observed for IgE (*p*=0.928) in male spray workers and non spray workers. Likewise, Non significant difference was also observed for IgE (*p*=0.425) in female spray workers and non spray workers as shown in **Table 3**. The increases in level of IgM indicated the chronic infection that caused the polyclonal hypergammaglobulinemia and the rise in immune response in spray workers. These discrepancies may be as a result of different components in pesticides mixtures such that only certain subclass of immunoglobulin was affected by each pesticide. No previous study in Pakistan reported information about the effect of pesticides exposure on the level of immunoglobulin. Undeger and Basaran (2001) reported that no change in serum IgG, IgA, IgM and C3 that was contradictory to current research. However Steerenberg *et al*. (2008) reported the increases in IgG4 levels and decrease in IgA level in European pesticides workers. Whereas Aroonvilairat *et al*. (2015) observed elevated level of total serum IgE unlike other immunoglobulin classes in Thai orchid farmers but it is not a single diagnostic condition for testing of pesticides toxicity in agriculture workers. Occupational exposure to multiple agricultural chemicals could be related to allergic rhinitis in Greece farmers (Chatzi *et al*., 2007; Fukuyama *et al*., 2009).

However, it was concluded that these alterations of some immunological and biochemical parameters was due to extensive toxicological pesticides effect on spray workers who frequently contacted with pesticides.

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